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Increased levels of HMGB-1 and endogenous secretory RAGE in induced sputum from asthmatic patients[☆]

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KEYWORDS

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Summary

Background: High mobility group box 1 (HMGB-1), a ligand of the receptor for advanced glycation end products (RAGE), is an inflammatory mediator in various disorders. Its endogenous decoy inhibitor, endogenous secretory RAGE (esRAGE), prevents the activation of RAGE signaling, and imbalance between HMGB-1 and esRAGE is known to be a factor determining progression of chronic inflammatory diseases.

Methods: We measured HMGB-1 and esRAGE levels in induced sputum from 44 asthmatic patients and 15 normal controls, and examined their correlations with asthma indices including pulmonary function test values and induced sputum indices.

Results: HMGB-1 levels in induced sputum were significantly higher in asthmatic patients than in normal controls ($p < 0.001$). Similarly, esRAGE levels were significantly higher in asthmatic patients than in normal controls ($p < 0.001$). In asthmatic patients, HMGB-1 levels were inversely correlated with percentage of predicted forced expiratory volume in 1 s (%FEV₁) and FEV₁/forced vital capacity (FEV₁/FVC). There was a significant increase in HMGB-1 level associated with severity of asthma ($p < 0.001$). However, there was no significant increase in esRAGE level associated with severity of asthma. In asthmatic patients, HMGB-1 levels were significantly correlated with percentage of neutrophils in induced sputum.

Abbreviations: AGEs, advanced glycation end products; esRAGE, endogenous secretory RAGE; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; HMGB-1, high mobility group box 1; ICAM-1, intercellular adhesion molecule-1; %FEV₁, percentage of predicted forced expiratory volume in 1 s; RAGE, receptor for advanced glycation end products; VCAM-1, vascular cell adhesion molecule-1.

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Conclusions: Our findings suggest that the HMGB-1 is a mediator of neutrophilic airway inflammation in asthma and that imbalance between HMGB-1 and esRAGE is related to the severity of asthma. Combined measurement of HMGB-1 and esRAGE may be novel biomarkers in asthma with severe airflow limitation.

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Introduction

Asthma is a chronic airway inflammatory disease associated with airway wall remodeling.¹ The airway inflammation in asthma is characterized by a complex immunopathology involving several different cell types and mediators, and inhaled corticosteroids (ICS) have been key therapeutic drugs in controlling this type of inflammation. The condition of some asthmatic patients is more severe and they consume much larger amounts of medical resources than those with less severe asthma. Severe asthma is strongly associated with death due to asthma. Particularly, asthma with increased risk of mortality was associated with the degree of airflow limitation.² It is thus essential to examine the characteristics of the asthma with severe airflow limitation and to determine new biomarkers and novel strategies for controlling it. Both resident and recruited inflammatory cells promote acute and chronic inflammation leading to persistent lung dysfunction due to structural remodeling. Many authors have reported that neutrophilic inflammation is involved in severe asthma.^{3–5} Neutrophilic airway inflammation is considered to induce airway damage leading to the development of the fixed airflow limitation, which is characteristic of severe asthma.⁶

A 30 kDa protein termed high mobility group box 1 (HMGB-1) was first purified from nuclei with histones.^{7,8} HMGB-1 is produced by many types of cells, and is located in both the cytoplasm and nucleus. Recently, HMGB-1 was found to be actively released in response to stimulation with endogenous proinflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and interferon (IFN)- γ from inflammatory and structural cells including activated monocytes, macrophages, and neutrophils.^{9–11} Moreover, HMGB-1 up-regulates the expression of endothelial adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) for inflammatory cell recruitment.¹² Consequently, HMGB-1 can mediate and enhance local and systemic inflammation. HMGB-1 has attracted much attention as an important mediator in inflammatory diseases such as atherosclerosis, arthritis, collagen disease, cancers, sepsis, and acute lung injury.^{13–19} HMGB-1 can bind to the receptor for advanced glycation end products (RAGE) in concentration-dependent fashion.²⁰ RAGE is a member of the immunoglobulin superfamily, and is expressed on inflammatory cells and many structural cells.^{21,22} RAGE interacts with a variety of ligands including advanced glycation end products (AGEs), amyloid peptide, S100 proteins and HMGB-1. Engagement of RAGE with its ligands activates various intracellular signaling pathways. Structural/functional studies revealed that a motif (amino acids 150–183) in the C-terminus of HMGB-1 was responsible for RAGE binding,²³ and extracellular HMGB-1-induced signaling

pathway activation has been reported to be inhibited by blocking antibodies to RAGE. Endogenous secretory RAGE (esRAGE) is a splice variant of RAGE and has a ligand-binding V-domain. esRAGE has ligand-binding activity and decoy function, and is known to be present in the circulation in humans.²⁴ The decoy function of esRAGE features a feedback mechanism by which esRAGE prevents the activation of RAGE signaling, and low circulating esRAGE levels are known to be a predictor of RAGE-mediated pathological conditions such as atherosclerosis and cardiovascular events in patients with end-stage renal disease.²⁵

However, the role of HMGB-1 in the pathogenesis of asthma and the involvement of the esRAGE feedback system in HMGB-1-mediated inflammatory responses in asthma are not yet fully understood. We hypothesized that the HMGB-1/RAGE signaling axis plays an important role in the pathogenesis of asthma and that the esRAGE feedback system plays a role in inhibiting the development of airway inflammation. To test this hypothesis, we examined HMGB-1 and esRAGE levels in induced sputum from asthmatic patients and normal controls and examined their correlations with asthma indices including pulmonary function test values and induced sputum indices.

Materials and methods

Subjects

A total of 44 asthmatic patients and 15 normal controls were enrolled in this study. All normal controls were healthy volunteers without history of lung diseases. All subjects were Japanese, non-smokers, and had no history of respiratory infection for at least the 4-week period preceding the study. All subjects gave written informed consent to participate in this study, which was approved by the Ethics Committee of Osaka City University (approval number: 1421). All asthmatic patients were newly diagnosed according to the American Thoracic Society (ATS) criteria for asthma²⁶ and were enrolled before asthma treatment including inhaled corticosteroids. Pulmonary function tests were performed for all subjects, and forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV₁) were measured. In all asthmatic patients except for subjects with severe airflow limitation (%FEV₁ is less than 70%), methacholine inhalation challenge testing was performed according to the recommendations of the ATS,²⁷ and they showed airway hyperresponsiveness to methacholine. All asthmatic patients exhibited airway reversibility to inhaled bronchodilator. The fraction of exhaled nitric oxide (NO) was also measured for all subjects using a chemiluminescence analyzer (SIEVER 280i NIPPON MEGACARE Co, Ltd, Tokyo, Japan) with a resolution of 1 part per billion (ppb) according to the recommendations of the ATS.²⁸ In addition, all patients

were subdivided into four categories by severity of asthma (intermittent, mild persistent, moderate persistent, severe persistent) based on symptoms, airflow limitation, and lung function variability according to the GINA 2006 guidelines,²⁹ as patients recruitment and data acquisition were done from 2007 to 2009.

Sputum induction and processing

Sputum induction was performed in accordance to the ERS guidelines as described previously.^{30,31} Spirometry was performed before inhalation of 200 µg salbutamol via a metered dose inhaler. All subjects were instructed to wash their mouth thoroughly with water. They then inhaled 3% saline at room temperature, nebulized via an ultrasonic nebulizer (NE-U12, Omron Co, Tokyo, Japan) at maximum output. They were encouraged to cough deeply at 3-min intervals, thereafter. After sputum induction, spirometry was repeated. If the FEV₁ declined, the subjects were required to wait until it returned to the baseline value. The volume of sputum samples was measured, and the samples were vortexed with phosphate-buffered solution (PBS). After that, the samples were divided into two portions. In one portion, dithiothreitol (DTT; final concentration of 1 mM) (WAKO Pure Chemical Industries Ltd, Osaka, Japan) was added and well vortexed and centrifuged at 400g for 10 min, and the cell pellet was resuspended. Slides were prepared by cytospin (Cytospin 3; Shandon, Tokyo, Japan) and stained by May-Grunwald-Giemsa for differential cell counts. In another portion of the sputum sample, for assay of HMGB-1 levels and esRAGE levels, no DTT was added to avoid potential confounding effects of DTT. Then the sputum supernatant diluted with PBS without DTT was ultracentrifuged at 60000g for 60 min at 4 °C to remove contaminating cellular debris. This was stored at -70 °C for subsequent assay of HMGB-1 levels and esRAGE levels. Both levels were measured in duplicated by quantitative sandwich enzyme immunoassays (HMGB-1 ELISA kit II; Shino-Test co, Kanagawa Japan, esRAGE ELISA kit; B-Bridge International, Inc., Sunnyvale CA USA), and we used the mean of the measurement values. As samples were diluted with PBS

in sputum processing, we recalculate original concentration by using dilution factor. All subjects produced an adequate specimen of sputum; a sample was considered adequate if the patient was able to expectorate at least 2 ml of sputum and if the slides contained less than 10% of squamous cells on differential cell counting.

Statistical analysis

All values were presented as medians (range). The comparisons between normal controls and asthmatic patients were analyzed by Mann–Whitney *U*-test. The comparisons among asthmatic groups subdivided by severity were analyzed by the Kruskal–Wallis test. When those comparisons were significant, the nonparametric analyses of variance with Bonferroni/Dunn's *post hoc* test were done. The significance of correlations was analyzed by Spearman's rank correlation coefficients. For all tests, findings of $p < 0.05$ were considered significant.

Results

The clinical characteristics of the study subjects are shown in Table 1. There was no significant difference in gender between asthmatic patients and normal controls. Median age of normal controls was significantly higher than that of asthmatic patients. There was no significant difference in age among the asthmatic subgroups. In contrast, %FEV₁ and FEV₁/FVC were significantly lower in asthmatic patients than those in normal controls (%FEV₁: $p < 0.01$; FEV₁/FVC: $p < 0.01$). Exhaled NO levels and percentages of eosinophils in induced sputum were significantly higher in asthmatic patients than those in normal controls ($p < 0.01$, $p < 0.01$, respectively). HMGB-1 levels in induced sputum were also significantly higher in asthmatic patients than those in normal controls (median (range): normal controls: 0 (0–45.2) ng/mL; asthmatic patients: 91.5 (0–590) ng/mL; $p < 0.001$, Fig. 1A). Similarly, esRAGE levels were significantly higher in asthmatic patients than those in normal controls (normal controls: 12.0 (0–31.9) pg/mL; asthmatic

Table 1 Clinical characteristics of the study subjects.

	Normal controls	Asthmatic patients	<i>p</i> -value
Subject number	15	44	—
Gender (M/F)	7/8	21/23	n.s.
Age	42 (31–54)	34 (23–51)	0.001
Asthma severity (1/2/3/4)	—	7/12/19/6	
FEV ₁ (% predicted)	106 (85–121)	78 (57–86)	<0.001
FEV ₁ /FVC (%)	86 (76–93)	68 (56–80)	<0.001
Exhaled NO (ppb)	16.5 (13–19)	32 (24–47)	<0.001
Induced sputum cell counts			
Eosinophils (%)	0.1 (0–0.5)	9.4 (4.5–15.5)	<0.001
Neutrophils (%)	19 (14–27)	24 (11–47)	<0.01
Macrophages (%)	76 (68–85)	60.5 (43–71)	<0.001
Lymphocytes (%)	0.9 (0.2–1.7)	1.55 (0.2–3.0)	0.003
Epithelial cells (%)	3.0 (0.5–5.8)	3.75 (1.3–7.2)	n.s.

All values are median (range).

FEV₁; forced expiratory volume in 1 s, FVC; forced vital capacity, Subject gender; M; male, F; female.

Asthma severity; 1; intermittent, 2; mild persistent, 3; moderate persistent, 4; severe persistent.

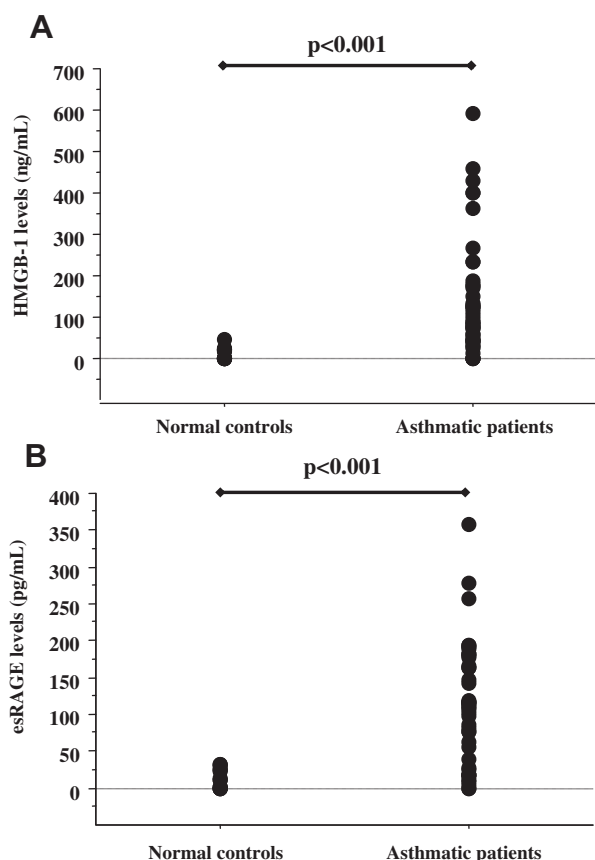


Figure 1 (A) Comparison of HMGB-1 levels in induced sputum of normal controls and asthmatic patients. (B) Comparison of esRAGE levels in induced sputum of normal controls and asthmatic patients.

patients: 110.5 (0–358) pg/mL; $p < 0.001$, Fig. 1B). We examined the correlations between HMGB-1 level and exhaled NO level in asthmatic patients. There was no significant correlation between HMGB-1 level and exhaled NO level ($r = -0.253$, $p = 0.098$). However, HMGB-1 level was significantly correlated with the degree of airflow limitation (FEV_1/FVC ; $r = -0.593$, $p < 0.001$, and $\%FEV_1$; $r = -0.758$, $p < 0.001$, Fig. 2).

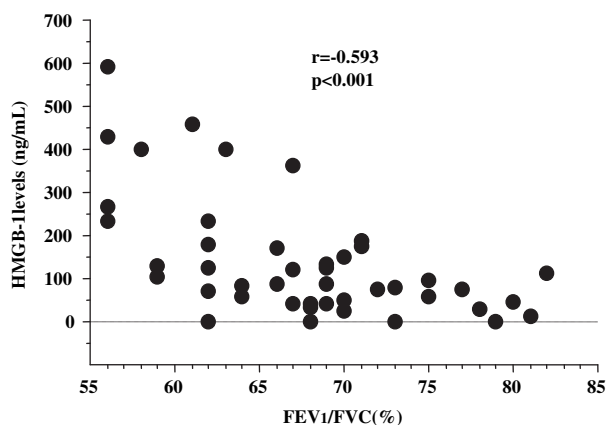


Figure 2 Correlation between HMGB-1 and FEV_1/FVC in asthmatic subjects.

We examined the correlations between percentages of neutrophils in induced sputum, HMGB-1 level and esRAGE level and severity of asthma. There were significant differences in percentages of neutrophils in induced sputum among the four subgroups of asthma ($p < 0.001$, Fig. 3A). In severe persistent asthmatic patients, there was significant increase in percentages of neutrophils. In asthmatic patients, there were also significant differences in HMGB-1 level among the four subgroups of asthma ($p < 0.001$, Fig. 3B). Although esRAGE levels in mild persistent to severe persistent asthmatic patients were significantly higher than those in normal controls ($p < 0.001$), there were no significant increase in esRAGE level associated with severity of asthma (Fig. 3C). We tested the correlations between HMGB-1 level and esRAGE level in normal controls, asthmatic patients and in each asthmatic subgroup with the different severity. However, there was no significant correlation between HMGB-1 level and esRAGE level. In asthmatic patients, there was a significant positive correlation between HMGB-1 level and percentage of neutrophils in induced sputum ($r = 0.729$, $p < 0.001$, Fig. 4), however, there was no significant correlation between esRAGE level and percentage of neutrophils.

Discussion

In this study, we found that HMGB-1 levels in induced sputum from asthmatic patients were significantly higher than those in induced sputum from normal controls. We also found that HMGB-1 level was significantly correlated with degree of airflow limitation. Moreover, we found that HMGB-1 level was increased in accordance with the severity of asthma. Exhaled NO is an easily measured marker of airway inflammation, and is thought to especially represent eosinophilic inflammation, a major feature of asthmatic airways. However, previous studies have demonstrated no significant difference in exhaled NO levels in asthmatics with moderate versus severe disease, suggesting sensitive biomarker for discriminating severe asthma from less severe asthma is needed.^{32–34} In this study, HMGB-1 was significantly elevated in accordance with airflow limitation, but not associated with exhaled NO levels.

In this study, esRAGE levels in induced sputum from asthmatic patients were significantly higher than those in normal controls. These findings suggest that an esRAGE feedback mechanism to prevent activation of the RAGE signaling cascade may be present in asthmatic airways. Decreased serum esRAGE levels are known to be associated with various pathological conditions such as atherosclerosis and cardiovascular events. When asthmatic patients were subdivided into four subgroups by severity, esRAGE levels differed significantly among the groups. The esRAGE levels in mild persistent to severe persistent asthmatic patients were significantly higher than those in normal controls. Although HMGB-1 levels were increased in accordance with the severity of asthma, there were no significant differences in esRAGE levels among the mild persistent to severe persistent asthmatic patients. Although the regulation and confounding factor of esRAGE expression in asthmatic airways is still not well understood, the esRAGE feedback mechanism might fail in asthmatic patients with more

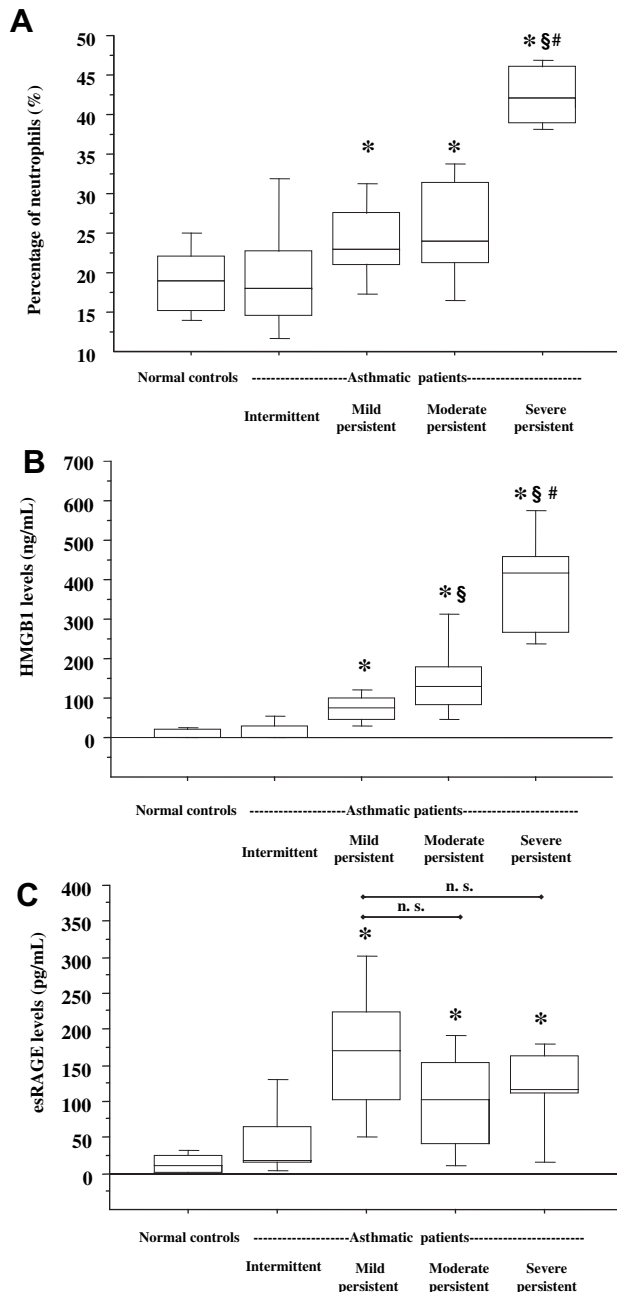


Figure 3 (A) Relationship between percentages of neutrophils in induced sputum and the severity of asthma. Values differed significantly among the five groups ($p < 0.001$); *versus normal controls, $p < 0.05$; §versus mild persistent asthmatic patients, $p < 0.001$; #versus moderate persistent asthmatic patients, $p < 0.001$. (B) Relationship between HMGB-1 level in induced sputum and the severity of asthma. Values differed significantly among the five groups ($p < 0.001$); *versus normal controls, $p < 0.05$; §versus mild persistent asthmatic patients, $p < 0.01$; #versus moderate persistent asthmatic patients, $p < 0.001$. (C) Relationship between esRAGE level in induced sputum and the severity of asthma. Values differed significantly among the five groups ($p < 0.001$); *versus normal controls, $p < 0.01$. Bars in the box represent the medians.

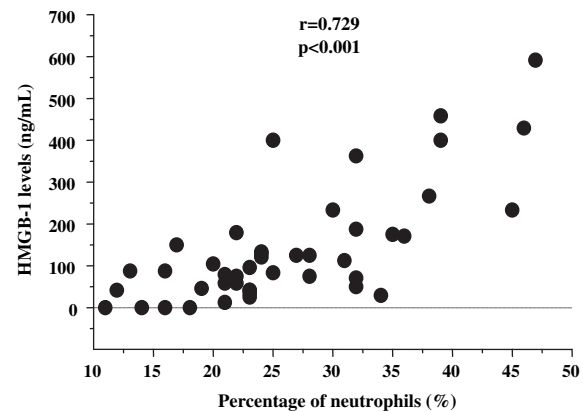


Figure 4 Correlation between HMGB-1 and percentage of neutrophils in induced sputum of asthmatic patients.

severe airflow limitation, such as those in moderate persistent or severe persistent. We simultaneously measured HMGB-1 and esRAGE levels and we found that esRAGE levels were not significantly increased in accordance with asthma severity. This discrepancy between HMGB-1 and esRAGE in severe asthma might be a cause of severe airflow limitation and could be used as biomarkers of severe asthma. Recently Smith and co-authors reported that plasma soluble RAGE were reduced in COPD patients, and was correlated with the severity of airflow limitation.³⁵ Their result suggests failure in negative feedback for RAGE signaling pathway is exists in severe chronic airway inflammatory disease and their result supports our results.

HMGB-1 is known to be a neutrophilic chemoattractant, so we examined the relationship between HMGB-1 and percentage of neutrophils in induced sputum of study subjects. As we shown, percentage of neutrophils in induced sputum in severe asthmatic patients is significantly higher compared with normal controls or less severe asthmatic patients. In these subgroups, there is a significant positive correlation between HMGB-1 level and the percentage of neutrophils in induced sputum. Recent studies have demonstrated that neutrophilic airway inflammation has a pathologically important role in the progression of persistent airflow limitation in severe asthma.⁴⁻⁶ HMGB-1 might work as a neutrophilic chemoattractant and involved in novel pathway of inflammation of severe asthma. The cellular source of HMGB-1 in induced sputum is unclear. Asthma is an inflammatory airway disease with involving a variety of inflammatory cells and recruited inflammatory cells might be one of the sources of HMGB-1. However, HMGB-1 is ubiquitously expressed in various types of cells and not only inflammatory cells, structural cells including epithelial or smooth muscle cell are the possible sources of HMGB-1 in the airway. In an *in vitro* model, HMGB-1 is passively released from necrotic cells/tissue or actively secreted from certain immune cells including macrophages, dendritic cells, and natural killer cells during infection.^{36,37} In human lungs, HMGB-1 is expressed in epithelial cells of airways and alveolar macrophages³⁸ and RAGE is highly expressed, particularly in pulmonary endothelium, bronchiolar epithelium, alveolar macrophages and type I alveolar epithelial cells.^{39,40} HMGB-1 and its receptor RAGE are co-localized in airways and

HMGB-1 might work in paracrine manner. Extracellularly transported HMGB-1 activates signaling pathways in endothelial or epithelial cells such as the NF- κ B signaling pathway and the mitogen-activated protein kinase (MAPK) through RAGE^{36,37} and thereby induces the production of neutrophilic chemotactic cytokines/chemokines such as TNF- α and IL-8.¹² Moreover, HMGB-1 also activates MAPKs (such as p38 and ERK1/2) and phosphatidylinositol 3-kinase/Akt and enhances the expression of proinflammatory cytokines in NF- κ B-dependent fashion in neutrophils.⁴¹ HMGB-1 augmented not only the neutrophil recruitment but also the activation of neutrophils. These findings suggest that the HMGB-1 signaling axis may induce neutrophil recruitment and activation in the airways and may play an important role in neutrophilic inflammation in asthmatic airways. HMGB-1 might thus be a new molecular target for controlling neutrophilic airway inflammation.

In conclusion, we showed for the first time that HMGB-1 and esRAGE levels in induced sputum were significantly higher from asthmatic patients than those in that from normal controls. HMGB-1 level increased in accordance with the severity of asthma, particularly the degree of airflow limitation, and was correlated with the percentage of neutrophils in induced sputum. esRAGE levels did not increase in accordance with the severity of asthma and this discrepancy between HMGB-1 and esRAGE might have important roles in neutrophilic inflammation in asthmatic airways. Combined measurement of HMGB-1 and esRAGE could be new biomarkers reflecting severity of asthma and neutrophilic inflammation in asthmatic airways. However, our study is small and preliminary and further studies addressing the roles of the HMGB-1/RAGE axis in asthmatic airway inflammation are needed to obtain novel strategies of treatment of asthma.

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Ethical approval

Ethical approval by the Ethics Committee of Osaka City University (approval number: 1421).

Conflicts of interest

None of the authors have any conflicts of interest to disclose.

References

- Jeffery PK. Remodeling in asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;**164**: S28–38.
- Panizza JA, James AL, Ryan G. Mortality and airflow obstruction in asthma: a 17-year follow-up study. *Intern Med J* 2006;**36**(12):773–80.
- Holgate ST. A need for circulating biomarkers of severe persistent asthma and its treatment. *Clin Exp Allergy* 2006;**36**(11):1355–6.
- Macdowell AL, Peters SP. Neutrophils in asthma. *Curr Allergy Asthma Rep* 2007;**7**(6):464–8.
- Holgate ST, Polosa R. The mechanisms, diagnosis, and management of severe asthma in adults. *Lancet* 2006;**368**(9537):780–93.
- Shaw DE, Berry MA, Hargadon B, et al. Association between neutrophilic airway inflammation and airflow limitation in adults with asthma. *Chest* 2007;**132**(6):1871–5.
- Wen L, Huang JK, Johnson BH, et al. A human placental cDNA clone that encodes nonhistone chromosomal protein HMG-1. *Nucleic Acids Res* 1989;**17**(3):1197–214.
- Ferrari S, Fineli P, Rocchi M, et al. The active gene that encodes human high mobility group 1 protein (HMG1) contains introns and maps to chromosome 13. *Genomics* 1996;**35**(2): 367–71.
- Wang H, Bloom O, Zhang M, et al. HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999;**285**(5425):248–51.
- Wang H, Vishnubhakat JM, Bloom O, et al. Proinflammatory cytokines (tumor necrosis factor and interleukin 1) stimulate release of high mobility group protein-1 by pituicytes. *Surgery* 1999;**126**(2):389–92.
- Rendon-Mitchell B, Ochani M, Li J, et al. IFN- γ induces high mobility group box 1 protein release partly through a TNF-dependent mechanism. *J Immunol* 2003;**170**(7):3890–7.
- Fiuzza C, Bustin M, Talwar S, et al. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. *Blood* 2003;**101**(7):2652–60.
- Hamada N, Maeyama T, Kawaguchi T, et al. The role of high mobility group box1 in pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2008;**39**(4):440–7.
- Kalinina N, Agrotis A, Antropova Y, et al. Increased expression of the DNA-binding cytokine HMGB-1 in human atherosclerotic lesions: role of activated macrophages and cytokines. *Arterioscler Thromb Vasc Biol* 2004;**24**:2320–5.
- Xiao XY, Lin L, Wen HP, et al. Increased serum HMGB1 level is associated with coronary artery disease in nondiabetic and type 2 diabetic patients. *Atherosclerosis* 2009;**205**:544–8.
- Wang H, Yang H, Tracey KJ. Extracellular role of HMGB1 in inflammation and sepsis. *J Intenal Med* 2004;**255**:320–31.
- Taira T, Matsuyama W, Mitsuyama H, et al. Increased serum high mobility group box-1 level in Churg-Strauss syndrome. *Clin Exp Immunol* 2007;**148**(2):241–7.
- Shang GH, Jia CQ, Tian H, et al. Serum high mobility group box protein 1 as a clinical marker for non-small cell lung cancer. *Respir Med* 2009;**103**(12):1949–53.
- Ueno H, Matsuda T, Hashimoto S, et al. Contributions of high mobility group box protein in experimental and clinical acute lung injury. *Am J Respir Crit Care Med* 2004;**170**(12):1310–6.
- Hori O, Brett J, Slatery T, et al. RAGE is a cellular binding site for amphotericin: mediation of neurite outgrowth and co-expression of RAGE and amphotericin in the developing nervous system. *J Biol Chem* 1995;**270**(43):25752–61.
- Schmidt AM, Vianna M, Gerlach M, et al. Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. *J Biol Chem* 1992;**267**(21):14987–97.
- Neeper M, Schmidt AM, Brett J, et al. Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *J Biol Chem* 1992;**267**(21):14998–5004.
- Huttunen HJ, Fages C, Kuja-Panula J, et al. Receptor for advanced glycation end products-binding COOH-terminal motif of amphotericin inhibits invasive migration and metastasis. *Cancer Res* 2002;**62**(16):4805–11.
- Yonekura H, Yamamoto Y, Sakurai S, et al. Novel splice variants of the receptor for advanced glycation end-products expressed

- in human vascular endothelial cells and pericytes, and their putative roles in diabetes-induced vascular injury. *J Biol Chem* 2003;**370**(Pt 3):1097–109.
25. Koyama H, Shoji T, Yokoyama H, et al. Plasma level of endogenous secretory RAGE is associated with components of the metabolic syndrome and atherosclerosis. *Arterioscler Thromb Vasc Biol* 2005;**25**(12):2587–93.
26. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, November 1986. *Am Rev Respir Dis* 1987;**136**(1):225–44.
27. American Thoracic Society. Standardization of spirometry, 1994 update. *Am J Respir Crit Care Med* 1995;**152**(3):1107–36.
28. Recommendations for standardized procedures for the on-line and off-line measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide in adults and children-1999. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. *Am J Respir Crit Care Med* 1999;**160**(6):2104–17.
29. *Global Initiative for asthma management and Prevention-GINA*. National Institutes Heart, Lung, and Blood Institute; 2006.
30. Paggiaro PL, Chanez P, Holz O, et al. Sputum induction. *Eur Respir J Suppl* 2002;**37**:3s–8s.
31. Kanazawa H, Asai K, Tochino Y, et al. Increased levels of angiopoietin-2 in induced sputum from smoking asthmatic patients. *Clin Exp Allergy* 2009;**39**(9):1330–7.
32. Kharitonov SA, Barnes PJ. Exhaled markers of pulmonary disease. *Am J Respir Crit Care Med* 2001;**163**(7):1693–722.
33. Lemiere C, Ernst P, Olivenstein R, et al. Airway inflammation assessed by invasive and noninvasive means in severe asthma: eosinophilic and noneosinophilic phenotypes. *J Allergy Clin Immunol* 2006;**118**(5):1033–9.
34. Tseliou E, Bessa V, Hillas G, et al. Exhaled nitric oxide and exhaled breath condensate pH in severe refractory asthma. *Chest* 2010;**138**(1):107–13.
35. Smith DJ, Yerkovich ST, Towers MA, et al. Reduced soluble receptor for advanced glycation end products in chronic obstructive pulmonary disease. *Eur Respir J*, in press.
36. Huttunen HJ, Kuja-Panila J, Sorci G, et al. Coregulation of neurite outgrowth and cell survival by amphotericin and S100 proteins through receptor for advanced glycation end products (RAGE) activation. *J Biol Chem* 2000;**275**(51):40096–105.
37. Degryse B, Bonaldi T, Scaffidi P, et al. The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. *J Cell Biol* 2001;**152**(6):1197–206.
38. Ferhani N, Letuve S, Kozhich A, et al. Expression of high-mobility group box 1 and of receptor for advanced glycation end products in COPD. *Am J Respir Crit Care Med* 2010;**181**(9):917–27.
39. Brett J, Schmidt AM, Yan SD, et al. Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. *Am J Pathol* 1993;**143**(6):1699–712.
40. Morbini P, Villa C, Campo I, et al. The receptor for advanced glycation end products and its ligands: a new inflammatory pathway in lung disease? *Mod Pathol* 2006;**19**(11):1437–45.
41. Park JS, Arcaroli J, Yum HK, et al. Activation of gene expression in human neutrophils by high mobility group box 1 protein. *Am J Physiol, Cell Physiol* 2003;**284**(4):C870–879.